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I further certify that pursuant to the provisions of Section 38(1) of the Patents Act 1990 a complete specification was filed on 17 April 2000 and it is an associated application to Provisional Application No. PP 9778 and has been allocated No. 779067.

WITNESS my hand this
Fifteenth day of May 2006

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AUSTRALIA

Patents Act 1990

MONASH UNIVERSITY

PROVISIONAL SPECIFICATION

Invention Title:

Improvement of T Cell Mediated Immunity

The invention is described in the following statement:

Improvement of T Cell Mediated Immunity

FIELD OF THE INVENTION

The present invention concerns methods of modifying the T-cell population make up or increasing the number of T-cells in a subject having depressed or abnormal T-cell population or function. These methods involve disrupting sex steroid signalling to the thymus in the subject.

BACKGROUND OF THE INVENTION

The thymus is influenced to a great extent by its bidirectional communication with the neuroendocrine system (Kendall, 1988). Of particular importance is the interplay between the pituitary, adrenals and gonads on thymic function including both trophic (TSH and GH) and atrophic effects (LH, FSH and ACTH) (Kendall, 1988; Homo-Delarche, 1991). Indeed one of the characteristic features of thymic physiology is the progressive decline in structure and function which is commensurate with the increase in circulating sex steroid production around puberty (Hirokawa and Makinodan, 1975; Tosi *et al.*, 1982 and Hirokawa, *et al.*, 1994). The precise target of the hormones and the mechanism by which they induce thymus atrophy is yet to be determined.. Since the thymus is the primary site for the production and maintenance of the peripheral T cell pool, this atrophy has been widely postulated as the primary cause of an increased incidence of immune-based disorders in the elderly. In particular, deficiencies of the immune system illustrated by a decrease in T-cell dependent immune functions such as cytolytic T-cell activity and mitogenic responses, are reflected by an increased incidence of immunodeficiency, autoimmunity and tumour load in later life (Hirokawa, 1998).

The impact of thymus atrophy is reflected in the periphery, with reduced thymic input to the T cell pool resulting in a less diverse T cell receptor (TCR) repertoire. Altered cytokine profile (Hobbs *et al.*, 1993; Kurashima *et al.*, 1995); changes in $CD4^+$ and $CD8^+$ subsets and a bias towards memory as opposed to naive T cells (Mackall *et al.*, 1995) are also observed. Furthermore, the efficiency of thymopoiesis is impaired with age such that the ability of the immune system to regenerate normal T-cell numbers after T-cell depletion, is eventually lost (Mackall *et al.*, 1995). However, recent work by Douek *et al.* (1998), has shown presumably thymic

output to occur even in old age in humans. Excisional DNA products of TCR gene-rearrangement were used to demonstrate circulating, *de novo* produced naive T cells after HIV infection in older patients. The rate of this output and subsequent peripheral T cell pool regeneration needs to be further addressed since patients who have undergone chemotherapy show a greatly reduced rate of regeneration of the T cell pool, particularly $CD4^+$ T cells, in post-pubertal patients compared to those who were pre-pubertal (Mackall et al, 1995). This is further exemplified in recent work by Timm and Thoman (1999), who have shown that although $CD4^+$ T cells are regenerated in old mice post BMT, they appear to show a bias towards memory cells due to the aged peripheral microenvironment, coupled to poor thymic production of naive T cells.

The thymus essentially consists of developing thymocytes interspersed within the diverse stromal cells (predominantly epithelial cell subsets) which constitute the microenvironment and provide the growth factors and cellular interactions necessary for the optimal development of the T cells. The symbiotic developmental relationship between thymocytes and the epithelial subsets that controls their differentiation and maturation (Boyd *et al.*, 1993), means sex-steroid inhibition could occur at the level of either cell type which would then influence the status of the other. It is less likely that there is an inherent defect within the thymocytes themselves since previous studies, utilising radiation chimeras, have shown that BM stem cells are not affected by age (Hirokawa, 1998; Mackall and Gress, 1997) and have a similar degree of thymus repopulation potential as young BM cells. Furthermore, thymocytes in older aged animals retain their ability to differentiate to at least some degree (Mackall and Gress, 1997; George and Ritter, 1996; Hirokawa *et al.*, 1994). However, recent work by Aspinall (1997), has shown a defect within the precursor $CD3^-CD4^-CD8^-$ triple negative (TN) population occurring at the stage of TCR β chain gene-rearrangement.

The enormous clinical benefits to be gained through restoration of thymic function, would represent an important strategy for the treatment of immunodeficiencies, particularly in the elderly, HIV patients and following chemotherapy.

SUMMARY OF THE INVENTION

The present inventors have demonstrated that thymic atrophy can be completely reversed by inhibition of sex steroid production, with full restoration of thymic structure and function. This serves as a basis for clinical applications of rejuvenating thymic function by disrupting sex steroid signalling to the thymus.

Accordingly, in a first aspect, the present invention provides a method of modifying the T-cell population makeup or increasing the number of T-cells in a subject having depressed or abnormal T-cell population or function, the method comprising disrupting sex steroid signalling to the thymus in the subject.

The phrase "modifying the T-cell population makeup" refers to altering the nature and/or ratio of T cell subsets defined functionally and by expression of characteristic molecules. Examples of these characteristic molecules includes, but is not limited to, the T cell receptor, CD4, CD8, CD3, CD25, CD28, CD44, CD62L and CD69.

The phrase "increasing the number of T-cells" refers to an absolute increase in the number of T cells in a subject in the thymus and/or in circulation and/or in the spleen and/or in the bone marrow and/or in peripheral tissues such as lymph nodes, gastrointestinal, urogenital and respiratory tracts. This phrase also refers to relative increase in T cells, for instance when compared to B cells.

A "subject having a depressed or abnormal T-cell population or function" includes an individual suffering from cancer, especially one who has undergone chemotherapy or radiation therapy, or has been subjected to a bone marrow transplant. This phrase also includes an individual infected with the human immunodeficiency virus, especially one who has AIDS. Furthermore, this phrase also includes any post-pubertal individual, especially an aged person who has decreased immune responsiveness and increased incidence of disease as a consequence of post-pubertal thymic atrophy. The phrase also includes individuals suffering from autoimmune or hypersensitivity diseases.

As will be appreciated, the present invention may be used to treat a subject suffering from an autoimmune disease. Autoimmune diseases are thought to arise as a polygenic trait, an essential component of which is the participation of pathological self reactive T cells. By treating such subjects

with chemotherapy or irradiation, with or without bone marrow transplantation, these self reactive T cells can be ablated. It is envisaged that disruption of sex steroid signalling to the thymus will allow reactivation of the thymus resulting in a cohort of new non-autoreactive T cells.

Accordingly, in a second aspect, the present invention provides a method for treating an autoimmune disease in a subject, the method comprising ablating the resident T cell population, and subsequently disrupting sex steroid signalling to the thymus in the subject.

In a preferred embodiment, this method further comprises subjecting the individual to a bone marrow transplant. In a further preferred embodiment, the T cell population is ablated by exposing the individual to chemotherapy or irradiation.

It will also be appreciated by those skilled in this field that the present invention may be utilized to enhance the efficiency of vaccination in subjects with depressed levels of T cells.

Accordingly, in a third aspect of the present invention provides a method for increasing an immune response to a vaccine in a subject, the method comprising disrupting sex steroid signalling to the thymus in the subject and administering a vaccine.

The vaccine may be, for example, against infectious agent(s) or against tumour antigens.

In preferred embodiments, the subject is suffering from cancer or an infection.

As will be readily understood, sex steroid signalling to the thymus can be disrupted in a range of ways, for example, inhibition of sex steroid production or blocking a sex steroid receptor(s) within the thymus. Inhibition of sex steroid production can be achieved, for example, by castration, administration of a sex steroid analogue(s), and other well known techniques. In a preferred embodiment, the sex steroid signalling to the thymus is disrupted by administration of a sex steroid analogue, preferably an analogue of luteinizing hormone-releasing hormone. It is currently preferred that the analogue is deslorelin (described in U.S. Patent No. 4218439).

Sex steroid analogues and their use in therapies and "chemical castration" are well known. Examples of such analogues include Eulexin (described in FR7923545, WO 86/01105 and PT100899), Goserelin (described

in US4100274, US4128638, GB9112859 and GB9112825), Leuprolide (described in US4490291, US3972859, US4008209, US4005063, DE2509783 and US4992421), dioxalan derivatives such as are described in EP 413209, Triptorelin (described in US4010125, US4018726, US4024121, EP 364819 and US5258492), Meterelin (described in EP 23904), Buserelin (described in US4003884, US4118483 and US4275001), Histrelin (described in EP217659), Nafarelin (described in US4234571, WO93/15722 and EP52510), Lutrelin (described in US4089946), Leuprorelin (described in Plosker *et al.*) and LHRH analogues such as are described in EP181236, US4608251, US4656247, US4642332, US4010149, US3992365 and US4010149. The disclosures of each the references referred to above are incorporated herein by cross reference.

In yet another preferred embodiment, the sex steroid analogue(s) is administered by a sustained peptide-release formulation. Preferred sustained peptide-release formulations are provided in WO 98/08533, the entire contents of which are incorporated herein by reference.

In another aspect, the present invention provides a method of decreasing host-vs-graft reaction in a subject following transplantation of an organ, the method comprising the following steps: (1) ablation of T-cells in the subject; (2) disrupting sex steroid signalling to the thymus of the subject; (3) transplanting bone marrow to the subject from a donor; and (4) transplanting the organ from the donor to the subject.

As will be understood, the term "organ" is used in its broadest sense and includes skin, kidney, liver, heart, lung etc.

Without wishing to be bound by scientific theory it is believed that in this aspect of the invention upon reactivation of the thymus, the donor marrow precursors will enter the thymus and develop in part into dendritic cells which will induce tolerance of the new T cells to the subsequent graft.

As will be understood by persons skilled in the art at least some of the means for disrupting sex steroid signalling to the thymus will only be effective as long as the appropriate compound is administered. As a result, an advantage of certain embodiments of the present invention is that once the desired immunological affects of the present invention have been achieved, (2-3 months) the treatment can be stopped and the subjects reproductive system will return to normal.

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

Brief Description of the Accompanying Figures

Fig. 1: Changes in thymocyte number pre- and post-castration. Thymus atrophy results in a significant decrease in thymocyte numbers with age. By 2 weeks post- castration, cell numbers have increased to young adult levels. By 3 weeks post-castration, numbers have significantly increased from the young adult and they are stabilised by 4 weeks post-castration. ***=Significantly different from young adult (2 mth) thymus, $p<0.001$

Fig. 2: (A) Spleen numbers remain constant with age and post-castration. The B:T cell ratio in the periphery also remains constant (B), however, the CD4:CD8 ratio decreases significantly ($p<0.001$) with age and is restored to normal young levels by 4 weeks post-ex.

Fig. 3: FACS profiles of CD4 vs. CD8 thymocyte populations with age and post-castration. Percentages for each quadrant are given above each plot. Subpopulations of thymocytes remain constant with age and there is a synchronous expansion of thymocytes following castration.

Fig. 4.1: Proliferation of thymocytes as detected by incorporation of a pulse of BrdU. Proportion of proliferating thymocytes remains constant with age and following castration.

Fig. 4.2: Effects of age and castration on proliferation of thymocyte subsets. (A) Proportion of each subset that constitutes the total proliferating population. The proportion of CD8+ T cells within the proliferating population is significantly increased. (B) Percentage of each subpopulation that is proliferating. The TN and CD8 Subsets have significantly less proliferation at 2 years than at 2 months. At 2 weeks post-castration, the TN population has returned to normal young levels of proliferation while the CD8 population shows a significant increase in proliferation. The level is

equivalent to the normal young by 4 weeks post-castration. (C) Overall TN proliferation remains constant with age and post-castration, however, the significant decrease in proliferation of the TN1 subpopulation with age, is not returned to normal levels by 4 weeks post-castration (D).***=Highly significant, $p<0.001$,**=significant, $p<0.01$

Fig. 5.1: Gross morphology of thymus of (A) 2 year old mouse 4 weeks post-castration; (B) normal 2 year old mouse or (C) normal 2 month old mouse. Note decline in thymic size with age and dramatic increase post-castration.

Fig. 5.2: Immunofluorescence of 2 month, 2 years and 2 weeks post-castration mouse thymus. Staining with MTS 10 (a) (medulla), anti-keratin (b) and MTS 10 with anti-keratin (c). Note the collapse of the 2 year thymus with indistinct corico-medullary junction and epithelial disruption (keratin).

Fig. 5.3: Immunofluorescence of thymus from 2 months, 2 years and 2 weeks post-castrated mice. Staining with MTS 44 (a) (cortex); anti-keratin (b) and MTS 44 with anti-keratin (c). Note the substantial decrease in cortical epithelium with age and rejuvenation post-cx.

Fig. 6: Migration rates from 1 year and 2 year mice as determined by IT FITC labelling. Young adult migration rates are 1% per day. Controls used were non-injected animals. Migration rates remain constant with age.

DETAILED DESCRIPTION OF THE INVENTION

Materials and Methods

Animals

CBA/CAH and C57Bl6/J male mice were obtained from Central Animal Services, Monash University and were housed under conventional conditions. Ages ranged from 4-6 weeks to 26 months of age and are indicated where relevant.

Castration

Animals were anaesthetised by intraperitoneal injection of 0.3ml of 0.3mg xylazine (Rompun; Bayer Australia Ltd., Botany NSW, Australia) and

1.5mg ketamine hydrochloride (Ketalar; Parke-Davis, Caringbah, NSW, Australia) in saline. Surgical castration was performed by a scrotal incision, revealing the testes, which were tied with suture and then removed along with surrounding fatty tissue.

Bromodeoxyuridine (BrdU) incorporation

Mice received two intraperitoneal injections of BrdU (Sigma Chemical Co., St. Louis, MO) (100mg/kg body weight in 100µl of PBS) at a 4 hour interval. Control mice received vehicle alone injections. One hour after the second injection, thymuses were dissected and either a cell suspension made for FACS analysis, or immediately embedded in Tissue Tek (O.C.T. compound, Miles INC, Indiana), snap frozen in liquid nitrogen, and stored at –70°C until use.

Flow Cytometric analysis

Mice were killed by CO_2 asphyxiation and thymus, spleen and mesenteric lymph nodes were removed. Organs were pushed gently through a 200µm sieve in cold PBS/1% FCS/0.02% Azide, centrifuged (650g, 5 min, 4°C), and resuspended in either PBS/FCS/Az. Spleen cells were incubated in red cell lysis buffer (8.9g/litre ammonium chloride) for 10 min at 4°C, washed and resuspended in PBS/FCS/Az. Cell concentration and viability were determined in duplicate using a haemocytometer and ethidium bromide/acridine orange and viewed under a fluorescence microscope (Axioskop; Carl Zeiss, Oberkochen, Germany).

For 3-colour immunofluorescence thymocytes were routinely labelled with anti-αβ TCR-FITC or anti-γδ TCR-FITC, anti-CD4-PE and anti-CD8-APC (all obtained from Pharmingen, San Diego, CA) followed by flow cytometry analysis. Spleen and lymph node suspensions were labelled with either αβTCR-FITC/CD4-PE/CD8-APC or B220-B (Sigma) with CD4-PE and CD8-APC. B220-B was revealed with streptavidin-Tri-color conjugate purchased from Caltag Laboratories, Inc., Burlingame, CA.

For BrdU detection, cells were surface labelled with CD4-PE and CD8-APC, followed by fixation and permeabilisation as previously described (Carayon and Bord, 1989). Briefly, stained cells were fixed O/N at 4°C in 1% PFA/0.01% Tween-20. Washed cells were incubated in 500µl DNase (100 Kunitz units, Boehringer Mannheim, W. Germany) for 30 mins at 37°C in order to denature the DNA. Finally, cells were incubated with anti-BrdU-FITC (Becton-Dickinson).

For 4-colour Immunofluorescence thymocytes were labelled for CD3, CD4, CD8, B220 and Mac-1, collectively detected by anti-rat Ig-Cy5 (Amersham, U.K.), and the negative cells (TN) gated for analysis. They were further stained for CD25-PE (Pharmingen) and CD44-B (Pharmingen) followed by Streptavidin-Tri-colour (Caltag, CA) as previously described (Godfrey and Zlotnik, 1993). BrdU detection was then performed as described above.

Samples were analysed on a FacsCalibur (Becton-Dickinson). Viable lymphocytes were gated according to 0° and 90° light scatter profiles and data was analysed using Cell quest software (Becton-Dickinson).

Immunohistology

Frozen thymus sections (4μm) were cut using a cryostat (Leica) and immediately fixed in 100% acetone.

For two-colour immunofluorescence, sections were double-labelled with a panel of monoclonal antibodies: MTS6, 10, 12, 15, 16, 20, 24, 32, 33, 35 and 44 (Godfrey *et al.*, 1990; Table 1) produced in this laboratory and the co-expression of epithelial cell determinants was assessed with a polyvalent rabbit anti-cytokeratin Ab (Dako, Carpinteria, CA). Bound mAb was revealed with FITC-conjugated sheep anti-rat Ig (Silenus Laboratories) and anti-cytokeratin was revealed with TRITC-conjugated goat anti-rabbit Ig (Silenus Laboratories).

For bromodeoxyuridine detection sections were stained with either anti-cytokeratin followed by anti-rabbit-TRITC or a specific mAb, which was then revealed with anti-rat Ig-Cy3 (Amersham). BrdU detection was then performed as previously described (Penit *et al.*, 1996). Briefly, sections were fixed in 70% Ethanol for 30 mins. Semi-dried sections were incubated in 4M HCl, neutralised by washing in Borate Buffer (Sigma), followed by two washes in PBS. BrdU was detected using anti-BrdU-FITC (Becton-Dickinson).

For three-colour immunofluorescence, sections were labelled for a specific MTS mAb together with anti-cytokeratin. BrdU detection was then performed as described above.

Sections were analysed using a Leica fluorescent and Nikon confocal microscopes.

Migration studies

Animals were anaesthetised by intraperitoneal injection of 0.3ml of 0.3mg xylazine (Rompun; Bayer Australia Ltd., Botany NSW, Australia) and 1.5mg ketamine hydrochloride (Ketalar; Parke-Davis, Caringbah, NSW, Australia) in saline.

Details of the FITC labelling of thymocytes technique are similar to those described elsewhere (Scollay *et al.*, 1980; Berzins *et al.*, 1998). Briefly, thymic lobes were exposed and each lobe was injected with approximately 10μm of 350 μg/ml FITC (in PBS). The wound was closed with a surgical staple, and the mouse was warmed until fully recovered from anaesthesia. Mice were killed by CO_2 asphyxiation approximately 24h after injection and lymphoid organs were removed for analysis.

After cell counts, samples were stained with anti-CD4-PE and anti-CD8-APC, then analysed by flow cytometry. Migrant cells were identified as live-gated $FITC^+$ cells expressing either CD4 or CD8 (to omit autofluorescing cells and doublets). The percentages of $FITC^+$ CD4 and CD8 cells were added to provide the total migrant percentage for lymph nodes and spleen, respectively. Calculation of daily export rates was performed as described by Berzins *et al.* (1998).

Data was analysed using the unpaired student 't' test or nonparametrical Mann-Whitney test was used to determine the statistical significance between control and test results for experiments performed at least in triplicate. Experimental values significantly differing from control values are indicated as follows: *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$.

Results

The effect of age on thymocyte populations.

(i) *Thymic weight and thymocyte number*

With increasing age there is a highly significant ($p \leq 0.0001$) decrease in both thymic weight (Fig. 1a) and total thymocyte number (Fig. 1b). Relative thymic weight (mg thymus/g body) in the young adult has a mean value of 3.34 which decreases to 0.66 at 18-24 months of age (adipose deposition limits accurate calculation). The decrease in thymic weight can be attributed to a decrease in total thymocyte numbers: the 1-2 month thymus contains $\sim 6.7 \times 10^7$ thymocytes, decreasing to $\sim 4.5 \times 10^6$ cells by 24 months. By removing the effects of sex steroids on the thymus by castration, regeneration occurs and by 4 weeks post-castration, the thymus is equivalent to that of the

young adult in both weight and cellularity (Fig. 1a and 1b). Interestingly, there is a significant ($p \leq 0.001$) increase in thymocyte numbers at 2 weeks post-castration ($\sim 1.2 \times 10^8$), which is restored to normal young levels by 4 weeks post-castration (Fig. 1b).

The decrease in T cell numbers produced by the thymus is not reflected in the periphery, with spleen cell numbers remaining constant with age (Fig. 2a). Homeostatic mechanisms in the periphery were evident since the B cell to T cell ratio in spleen and lymph nodes was not affected with age and the subsequent decrease in T cell numbers reaching the periphery (Fig. 2b). However, the ratio of $CD4^+$ to $CD8^+$ T cell significantly decreased ($p \leq 0.001$) with age from 2:1 at 2 months of age, to a ratio of 1:1 at 2 years of age (Fig. 2c). Following castration and the subsequent rise in T cell numbers reaching the periphery, no change in peripheral T cell numbers was observed: splenic T cell numbers and the ratio of B:T cells in both spleen and lymph nodes was not altered following castration (Fig. 2a-b). The decreased CD4:CD8 ratio in the periphery with age was still evident at 2 weeks post-castration but was completely reversed by 4 weeks post-castration (Fig. 2c).

(ii) *αβTCR, γδTCR, CD4 and CD8 expression*

To determine if the decrease in thymocyte numbers seen with age was the result of the depletion of specific cell populations, thymocytes were labelled with defining markers in order to analyse the separate subpopulations. In addition, this allowed analysis of the kinetics of thymus repopulation post-castration. The proportion of the main thymocyte subpopulations was compared with those of the normal young thymus (Fig. 3) and found to remain uniform with age. In addition, further subdivision of thymocytes by the expression of αβTCR and γδTCR revealed no change in the proportions of these populations with age (data not shown). At 2 and 4 weeks post-castration, thymocyte subpopulations remained in the same proportions and, since thymocyte numbers increase by up to 100-fold post-castration, this indicates a synchronous expansion of all thymocyte subsets rather than a developmental progression of expansion.

The decrease in cell numbers seen in the thymus of aged animals thus appears to be the result of a balanced reduction in all cell phenotypes, with no significant changes in T cell populations being detected. Thymus

regeneration occurs in a synchronous fashion, replenishing all T cell subpopulations simultaneously rather than sequentially.

Proliferation of thymocytes

As shown in Fig. 4.1, 15-20% of thymocytes are proliferating at 4-6 weeks of age. The majority (~80%) of these are DP with the TN subset making up the second largest population at ~6% (Fig. 4.2A). Accordingly, most division is seen in the subcapsule and cortex by immunohistology (Fig 5.1A). Some division is seen in the medullary regions with FACS-analysis revealing a proportion of SP cells (9% of CD4 T cells and 25% of CD8 T cells) dividing (Fig. 4.2B).

Although cell numbers are significantly decreased in the aged thymus, proliferation of thymocytes remains constant, decreasing to 12-15% at 2 years (Fig 4.1), with the phenotype of the proliferating population resembling the 2 month thymus (Fig. 4.2A). Immunohistology revealed the division at 1 year of age to reflect that seen in the young adult, however, at 2 years, proliferation is mainly seen in the outer cortex and surrounding the vasculature (Fig. 5.1b). At 2 weeks post-castration, although thymocyte numbers significantly increase, there is no change in the proportion of thymocytes that are proliferating, again indicating a synchronous expansion of cells (Fig. 4.1). Immunohistology revealed the localisation of thymocyte proliferation and the extent of dividing cells to resemble the situation in the 2-month-old thymus by 2 weeks post-castration (Fig. 5.1c). When analysing the proportion of each subpopulation which represent the proliferating population, there was a significant ($p<0.001$) increase in the percentage of CD8 T cells which are within the proliferating population (1% at 2 months and 2 years of age, increasing to ~6% at 2 weeks post-castration) (Fig. 4.2A).

Fig. 4.2B illustrates the extent of proliferation within each subset in young, old and castrated mice. There is a significant ($p\leq0.001$) decay in proliferation within the DN subset (35% at 2 months to 4% by 2 years). Proliferation of $CD8^+$ T cells was also significantly ($p\leq0.001$) decreased, reflecting the findings by immunohistology (Fig.5.1B) where no division is evident in the medulla of the aged thymus. The decrease in DN proliferation is not returned to normal young levels by 4 weeks post-castration. However, proliferation within the $CD8^+$ T cell subset is significantly ($p\leq0.001$)

increased at 2 weeks post-castration and is returning to normal young levels at 4 weeks post-castration.

The decrease in proliferation within the DN subset was analysed further using the markers CD44 and CD25. The DN subpopulation, in addition to the thymocyte precursors, contains $\alpha\beta TCR^{+}CD4^{-}CD8^{-}$ thymocytes, which are thought to have downregulated both co-receptors at the transition to SP cells (Godfrey & Zlotnik, 1993). By gating on these mature cells, it was possible to analyse the true TN compartment ($CD3^{-}CD4^{-}CD8^{-}$) and these showed no difference in their proliferation rates with age or following castration (Fig. 4.2C). However, analysis of the subpopulations expressing CD44 and CD25, showed a significant ($p<0.001$) decrease in proliferation of the TN1 subset ($CD44^{+}CD25^{-}$), from 20% in the normal young to around 6% at 18 months of age (Fig. 4.2D) which was restored by 4 weeks post-castration. The decrease in the proliferation of the TN1 subset, was compensated for by a significant ($p\leq0.001$) increase in proliferation of the TN2 subpopulation ($CD44^{+}CD25^{+}$) which returned to normal young levels by 2 weeks post-castration (Fig. 4.2D).

The effect of age on the thymic microenvironment.

The changes in the thymic microenvironment with age were examined by immunofluorescence using an extensive panel of mAbs from the MTS series, double-labelled with a polyclonal anti-cytokeratin Ab.

The antigens recognised by these mAbs can be subdivided into three groups: thymic epithelial subsets, vascular-associated antigens and those present on both stromal cells and thymocytes.

(I) *Epithelial cell antigens.*

Anti-keratin staining (pan-epithelium) of 2 year old mouse thymus, revealed a loss of general thymus architecture with a severe epithelial cell disorganisation and absence of a distinct cortico-medullary junction (Fig. 5.2 A and B). Further analysis using the mAbs, MTS 10 (medulla, Fig. 5.3a and b) and MTS44 (cortex, Fig. 5.4), showed a distinct reduction in cortex size with age, with a less substantial decrease in medullary epithelium. Epithelial cell free regions, or keratin negative areas (KNA's, van Ewijk *et al.*, 1980; Godfrey *et al.*, 1990; Bruijntjes *et al.*, 1993).) were more apparent and increased in size in the aged thymus, as evident with anti-cytokeratin labelling (Fig. 5.1). There is also the appearance of thymic epithelial "cyst-like" structures in the aged thymus particularly noticeable in medullary

regions (Fig. 5.1). Adipose deposition, severe decrease in thymic size and the decline in integrity of the cortico-medullary junction are shown conclusively with the anti-cytokeratin staining (Fig. 5.1). As shown in Fig. 2.1, the thymus is beginning to regenerate by 2 weeks post-castration. This is evident in the size of the thymic lobes (a), the increase in cortical epithelium as revealed by MTS 44 (b) and the localisation of medullary epithelium (c). The medullary epithelium is detected by MTS 10 and at 2 weeks, there are still subpockets of epithelium stained by MTS 10 scattered throughout the cortex. By 4 weeks post-castration, there is a distinct medulla and cortex and discernible cortico-medullary junction (Fig. 5.1c and d).

The markers MTS 20 and 24 are presumed to detect primordial epithelial cells (Godfrey, *et al.*, 1990) and further illustrate the degeneration of the aged thymus. These are present in abundance at E14, detect isolated medullary epithelial cell clusters at 4- 6 weeks but are again increased in intensity in the aged thymus (Fig 5.5a). Following castration, all these antigens are expressed at a level equivalent to that of the young adult thymus (Figs. 5b) with MTS 20 and MTS 24 reverting to discrete subpockets of epithelium located at the cortico-medullary junction.

(ii) *Vascular-associated antigens.*

The blood-thymus barrier is thought to be responsible for the immigration of T cell precursors to the thymus and the emigration of mature T cells from the thymus to the periphery.

The mAb MTS 15 is specific for the endothelium of thymic blood vessels, demonstrating a granular, diffuse staining pattern (Godfrey, *et al,* 1990). In the aged thymus, MTS 15 expression is greatly increased, and reflects the increased frequency and size of blood vessels and perivascular spaces (data not shown).

The thymic extracellular matrix, containing important structural and cellular adhesion molecules such as collagen, laminin and fibrinogen, is detected by the mAb MTS 16. Scattered throughout the normal young thymus, the nature of MTS 16 expression becomes more widespread and interconnected in the aged thymus (Fig. 5.6 a and b). Expression of MTS 16 is increased further at 2 weeks post-castration (Fig. 5.6c) while 4 weeks post-castration, this expression is representative of the situation in the 2 month thymus (Fig. 5.6d).

(iii) *Shared antigens*

MHC II expression in the normal young thymus, detected by the mAb MTS 6, is strongly positive (granular) on the cortical epithelium (Fig. 5.7a, Godfrey *et al.*, 1990) with weaker staining of the medullary epithelium. The aged thymus shows a decrease in MHCII expression (Fig. 5.7b) with expression substantially increased at 2 weeks post-castration (Fig. 5.7c). By 4 weeks post-castration, expression is again reduced and appears similar to the 2 month old thymus (Fig. 5.7d).

Thymocyte emigration

Approximately 1% of T cells migrate from the thymus daily in the young mouse (Scollay *et al.*, 1980). We found migration was occurring at a proportional rate equivalent to the normal young mouse at 14 months and even 2 years of age (Fig.6) although significantly ($p \leq 0.0001$) reduced in number. There was an increase in the CD4:CD8 ratio of the recent thymic emigrants from ~3:1 at 2 months to ~7:1 at 26 months. By 1 week post-castration, cell number migrating to the periphery has substantially increased with the overall rate of migration remaining constant at 1-1.5%.

Discussion

The present invention discloses that the aged thymus, although severely atrophic, maintains its functional capacity with age, with T cell proliferation, differentiation and migration occurring at levels equivalent to the young adult mouse. Although thymic function is regulated by several complex interactions between the neuro-endocrine-immune axes, the atrophy induced by sex steroid production exerts the most significant and prolonged effects illustrated by the extent of thymus regeneration post-castration both of lymphoid and epithelial cell subsets.

Thymus weight is significantly reduced with age as shown previously (Hirokawa and Makinodan, 1975, Aspinall, 1997) and correlates with a significant decrease in thymocyte numbers. The stress induced by the castration technique, which may result in further thymus atrophy due to the actions of corticosteroids, is overridden by the removal of sex steroid influences with the 2-week castrate thymus increasing in cellularity by 20-30 fold from the pre-castrate thymus. By 3 weeks post-castration, the aged thymus shows a significant increase in both thymic size and cell number, surpassing that of the young adult thymus presumably due to the actions of sex steroids already exerting themselves in the 2 month old mouse.

Our data confirms previous findings that emphasise the continued ability of thymocytes to differentiate and maintain constant subset proportions with age (Aspinall, 1997). In addition, we have shown thymocyte differentiation to occur simultaneously post-castration indicative of a synchronous expansion in thymocyte subsets. Since thymocyte numbers are decreased significantly with age, proliferation of thymocytes was analysed to determine if this was a contributing factor in thymus atrophy.

Proliferation of thymocytes was not affected by age-induced thymic atrophy or by removal of sex-steroid influences post-castration with ~14% of all thymocytes proliferating. However, the localisation of this division differed with age: the 2 month mouse thymus shows abundant division throughout the subcapsular and cortical areas (TN and DP T cells) with some division also occurring in the medulla. Due to thymic epithelial disorganisation with age, localisation of proliferation was difficult to distinguish but appeared to be less uniform in pattern than the young and relegated to the outer cortex. By 2 weeks post-castration, dividing thymocytes were detected throughout the cortex and were evident in the medulla with similar distribution to the 2 month thymus.

The phenotype of the proliferating population as determined by CD4 and CD8 analysis, was not altered with age or following castration. However, analysis of proliferation within thymocyte subpopulations, revealed a significant decrease in proliferation of both the TN and $CD8^+$ cells with age. Further analysis within the TN subset on the basis of the markers CD44 and CD25, revealed a significant decrease in proliferation of the TN1 ($CD44^+CD25^-$) population which was compensated for by an increase in the TN2 ($CD44^-CD25^+$) population. These abnormalities within the TN population, reflect the findings by Aspinall (1997). Surprisingly, the TN subset was proliferating at normal levels by 2 weeks post-castration indicative of the immediate response of this population to the inhibition of sex-steroid action. Additionally, at both 2 weeks and 4 weeks post-castration, the proportion of $CD8^+$ T cells that were proliferating was markedly increased from the control thymus, possibly indicating a role in the re-establishment of the peripheral T cell pool.

Thymocyte migration was shown to occur at a constant proportion of thymocytes with age conflicting with previous data by Scollay et al (1980) who showed a ten-fold reduction in the rate of thymocyte migration to the

periphery. The difference in these results may be due to the difficulties in intrathymic FITC labelling of 2 year old thymuses or the effects of adipose deposition on FITC uptake. However, the absolute numbers of T cells migrating was decreased significantly as found by Scollay resulting in a significant reduction in ratio of RTEs to the peripheral T cell pool. This will result in changes in the periphery predominantly affecting the T cell repertoire (Mackall *et al.*, 1995). Previous papers (Mackall et al, 1995) have shown a skewing of the T cell repertoire to a memory rather than naive T cell phenotype with age. The diminished T cell repertoire however, may not cope if the individual encounters new pathogens, possibly accounting for the rise in immunodeficiency in the aged. Obviously, there is a need to re-establish the T cell pool in immunocompromised individuals. Castration allows the thymus to repopulate the periphery through significantly increasing the production of naive T cells.

In the periphery, T cell numbers remained at a constant level as evidenced in the B:T cell ratios of spleen and lymph nodes, presumably due to peripheral homeostasis (Mackall *et al.*, 1995; Berzins *et al.*, 1998). However, disruption of cellular composition in the periphery was evident with the aged thymus showing a significant decrease in CD4:CD8 ratios from 2:1 in the young adult to 1:1 in the 2 year mouse, possibly indicative of the more susceptible nature of $CD4^+$ T cells to age or an increase in production of $CD8^+$ T cells from extrathymic sources. By 2 weeks post-castration, this ratio has been normalised, again reflecting the immediate response of the immune system to surgical castration.

The above findings have shown firstly that the aged thymus is capable of functioning in a nature equivalent to the pre-pubertal thymus. In this respect, T cell numbers are significantly decreased but the ability of thymocytes to differentiate is not disturbed. Their overall ability to proliferate and eventually migrate to the periphery is again not influenced by the age-associated atrophy of the thymus. However, two important findings were noted. Firstly, there appears to be an adverse affect on the TN cells in their ability to proliferate, correlating with findings by Aspinall (1997). This defect could be attributed to an inherent defect in the thymocytes themselves. Yet our data, and previous work has shown thymocyte differentiation, although diminished, still occurs and stem cell entry from the BM is also not affected with age (Hirokawa, 1998; Mackall and Gress, 1997).

This implicates the thymic stroma as the target for sex steroid action and consequently abnormal regulation of this precursor subset of T cells. Secondly, the $CD8^+$ T cells were significantly diminished in their proliferative capacity with age and, following castration, a significantly increased proportion of $CD8^+$ T cells proliferated as compared to the 2 month mouse. The proliferation of mature T cells is thought to be a final step before migration (Suda and Zlotnik, 1992), such that a significant decrease in $CD8^+$ proliferation would indicate a decrease in their migrational potential. This hypothesis is supported by our finding that the ratio of CD4:CD8 T cells in RTEs increased with age, indicative of a decrease in CD8 T cells migrating. Alternatively, if the thymic epithelium is providing the key factor for the CD8 T cell maintenance, whether a lymphostromal molecule or cytokine influence, this factor may be disturbed with increased sex-steroid production. By removing the influence of sex-steroids, the CD8 T cell population can again proliferate optimally. Thus, it was necessary to determine, in detail, the status of thymic epithelial cells pre- and post-castration.

The cortex appears to 'collapse' with age due to lack of thymocytes available to expand the network of epithelium. The most dramatic change in thymic epithelium post-castration was the increased network of cortical epithelium detected by MTS 44, illustrating the significant rise in thymocyte numbers. At 2 weeks post-castration, KNAs are abundant and appear to accommodate proliferating thymocytes indicating that thymocyte development is occurring at a rate higher than the epithelium can cope with. The increase in cortical epithelium appears to be due to stretching of the thymic architecture rather than proliferation of this subtype since no proliferation of the epithelium was noted with BrdU staining by immunofluorescence.

Medullary epithelium is not as susceptible to age influences most likely due to the lesser number of T cells accumulating in this area (>95% of thymocytes are lost at the DP stage due to selection events). However, the aged thymus shows severe epithelial cell disruption distinguished by a lack of distinction of the cortico-medullary junction with the medullary epithelium incorporating into the cortical epithelium. By 2 weeks post-castration, the medullary epithelium, as detected by MTS 10 staining is re-organised to some extent, however, subpockets are still present within the cortical epithelium. By 4 weeks post-castration, the cortical and medullary

epithelium is completely reorganised with a distinct cortico-medullary junction similar to the young adult thymus.

Subtle changes were also observed following castration, most evident in the decreased expression of MHC class II and blood-thymus barrier antigens when compared to the pre-castrate thymus. MHCII (detected by MTS6) is increased in expression in the aged thymus possibly relating to a decrease in control by the developing thymocytes due to their diminished numbers. Alternatively, it may simply be due to lack of masking by the thymocytes, illustrated also in the post-irradiation thymus (Randle and Boyd, 1992) which is depleted of the DP thymocytes. Once thymocyte numbers are increased following castration, the antigen binding sites are again blocked by the accumulation of thymocytes thus decreasing detection by immunofluorescence. The antigens detecting the blood-thymus barrier (MTS12, 15 and 16) are again increased in the aged thymus and also revert to the expression in the young adult thymus post-castration. Lack of masking by thymocytes and the close proximity of the antigens due to thymic atrophy may explain this increase in expression. Alternatively, the developing thymocytes may provide the necessary control mechanisms over the expression of these antigens thus when these are depleted, expression is not controlled. The primordial epithelial antigens detected by MTS 20 and MTS 24 are increased in expression in the aged thymus but revert to subpockets of epithelium at the cortico-medullary junction post-castration. This indicates a lack of signals for this epithelial precursor subtype to differentiate in the aged mouse. Removing the block placed by the sex-steroids, these antigens can differentiate to express cortical epithelial antigens.

The above findings indicate a defect in the thymic epithelium rendering it incapable of providing the developing thymocytes with the necessary stimulus for development. However, the symbiotic nature of the thymic epithelium and thymocytes makes it difficult to ascertain the exact pathway of destruction by the sex steroid influences. The medullary epithelium requires cortical T cells for its proper development and maintenance. Thus, if this population is diminished, the medullary thymocytes may not receive adequate signals for development. This particularly seems to affect the $CD8^+$ population. $IRF^{-/-}$ mice show a decreased number of $CD8^+$ T cells. It would therefore, be interesting to determine the proliferative capacity of these cells.

The defect in proliferation of the TN1 subset which was observed indicates that loss of cortical epithelium affects thymocyte development at the crucial stage of TCR gene rearrangement whereby the cortical epithelium provides factors such as IL-7 and SCF necessary for thymopoiesis (Godfrey and Zlotnik, 1990; Aspinall, 1997). Indeed, IL-$7^{-/-}$ and IL-$7R^{-/-}$ mice show similar thymic morphology to that seen in aged mice (Wiles *et al.*, 1992; Zlotnik and Moore, 1995; von Freeden-Jeffry, 1995). Further work is necessary to determine the changes in IL-7 and IL-7R with age.

In conclusion, the aged thymus still maintains its functional capacity, however, the thymocytes that develop in the aged mouse are not under the stringent control by thymic epithelial cells as seen in the normal young mouse due to the lack of structural integrity of the thymic microenvironment. Thus the proliferation, differentiation and migration of these cells will not be under optimal regulation and may result in the increased release of autoreactive/immunodysfunctional T cells in the periphery. The defects within both the TN and particularly, $CD8^+$ populations, may result in the changes seen within the peripheral T cell pool with age. In addition, we have described in detail, the effects of castration on thymic epithelial cell development and reorganisation. The mechanisms underlying thymic atrophy utilising steroid receptor binding assays and the role of thymic epithelial subsets in thymus regeneration post-castration are currently under study. Restoration of thymus function by castration will provide an essential means for regenerating the peripheral T cell pool and thus in re-establishing immunity in immunosuppressed individuals.

SUMMARY

In summary, in the method of the present invention, restoration of thymic function and hence cell-mediated immunity will be achieved by distrupting sex steroid signalling to the thymus. This will be achieved mainly through application of LHRH analogues using surgical implants. In some clinical cases this may be permanent removal of the gonads via physical castration. Some examples of the conditions in which the method of the present invention may be applicable are:

(i) Endometriosis

(ii) Cancer patients particularly breast and prostate, but including any cancers or proliferative disorders resulting in T cell abnormalities or reduced functional capacity of cell-mediated immunity.

(iii) Allogeneic BM transplantation.

(iv) Post-chemotherapy leukaemia patients. This would include CLL and low grade Non-Hogkins lymphoma patients treated with drugs such as Fludarabine, cladrabine, dexamethasone and 2-cytodeoxyadenosine which are severely toxic for T cells.

(v) Patients with immunodeficiencies such as HIV AIDS.

(vi) Immune dysfunction including immunodeficiencies in the elderly.

(vii) Autoimmune disease the treatment for which would include lymphocyte depletion (eg through irradiation or chemotherapy) followed by bone marrow/stem cell transplantation linked with LHRH.

(viii) Organ and tissue including skin transplants - host lymphocytes would be first depleted (eg through irradiation or chemotherapy). This could be followed by donor bone marrow/stem cell transplantation linked with LHRH to establish chimeras which would include establishment of donor cells including dendritic cells in the host thymus to cause tolerance of newly developed host T cells to the donor. After establishment of the central tolerance the host would receive a graft from the donor of the stem cells.

(ix) Normalising of T cell abnormalities in allergies or hypersensitivities.

(x) Improvement of vaccinations of all types through increases in T cells.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

Dated this fifteenth day of April 1999

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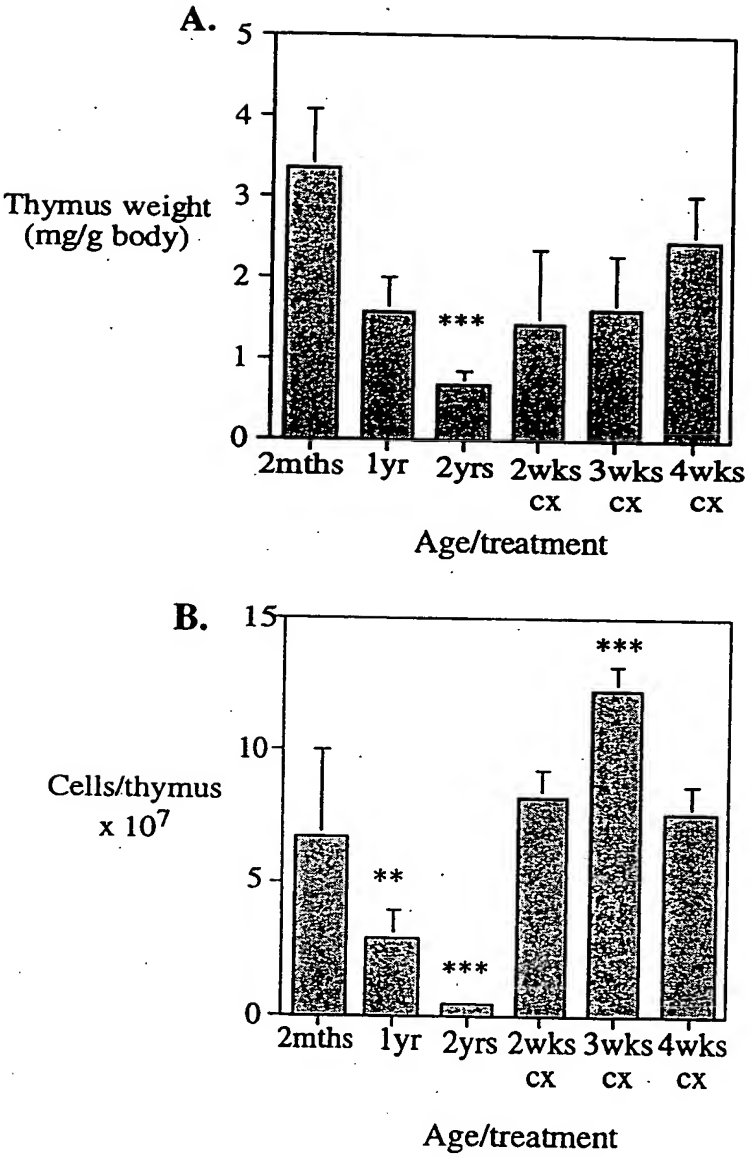

Fig. 1: Changes in thymocyte number pre- and post- castration. Thymus atrophy results in a significant decrease in thymocyte numbers with age. By 2 weeks post-castration, cell numbers have increased to young adult levels. By 3 weeks post-castraton, numbers have significantly increased from the young adult and they are stabilised by 4 weeks post-castration.

*** = significantly different from young adult (2 mth) thymus, $p<0.001$

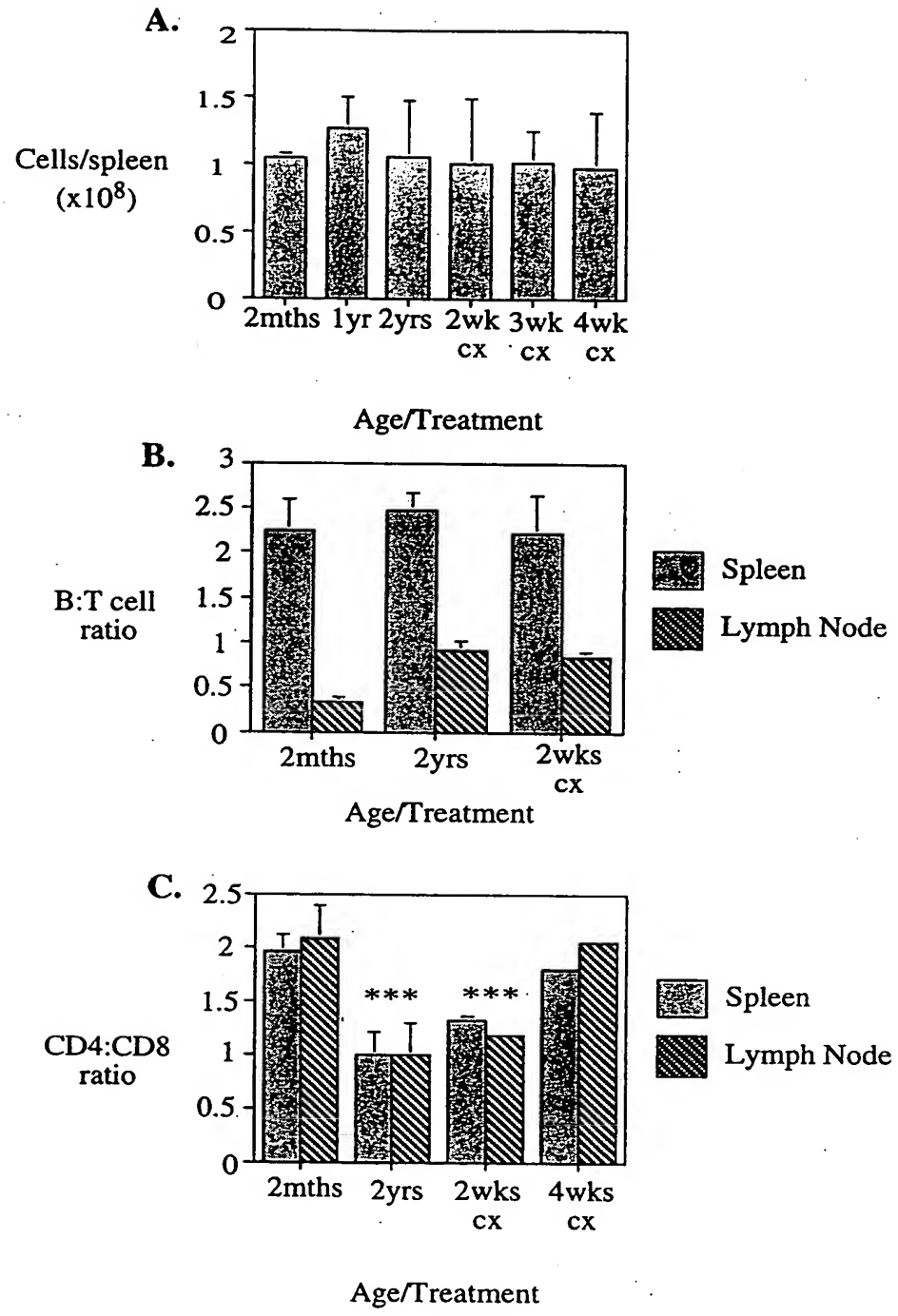

Fig. 2: (A) Spleen cell numbers remain constant with age and post-castration. The B:T cellratio in the periphery also remains constant (B), however, the CD4:CD8 ratio decreases significantly ($p<0.001$)with age and is restored to normal young levels by 4 weeks post-cx.

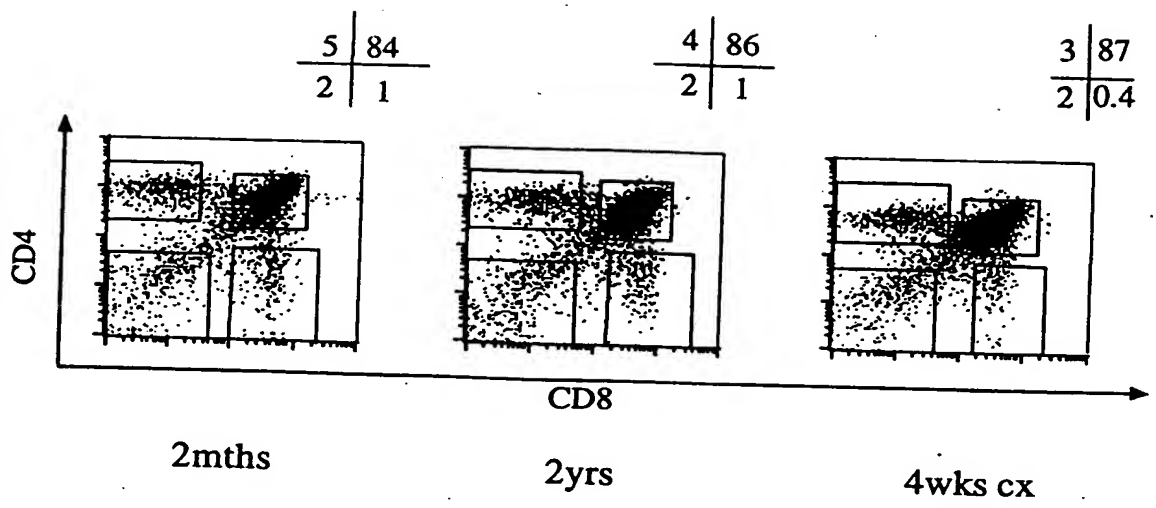

Fig. 3: FACS profiles of CD4 vs. CD8 thymocyte populations with age and post-castration. Percentages for each quadrant are given above each plot. Subpopulations of thymocytes remain constant with age and there is a synchronous expansion of thymocytes following castration.

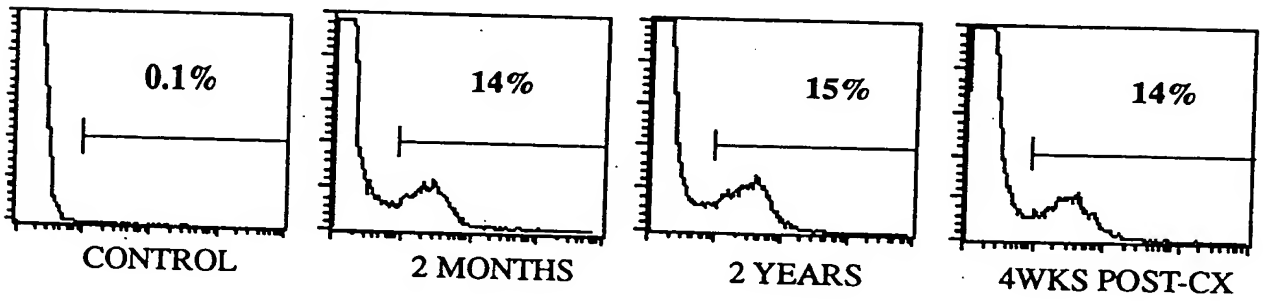

Fig. 4.1: Proliferation of thymocytes as detected by incorporation of a pulse of BrdU. Proportion of proliferating thymocytes remains constant with age and following castration.

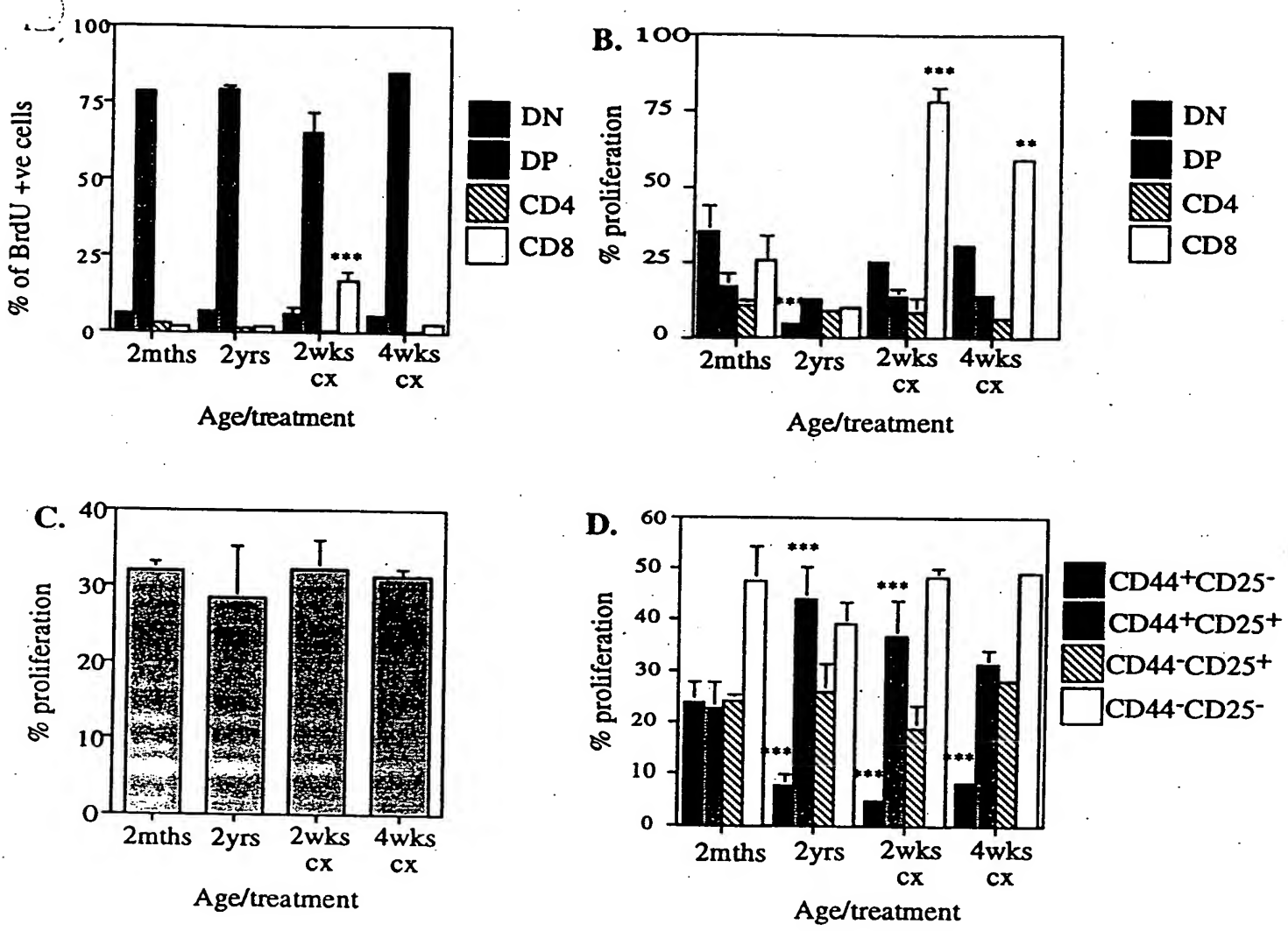

Fig. 4.2: Effects of age and castration on proliferation of thymocyte subsets. (A) Proportion of each subset that constitutes the total proliferating population. The proportion of $CD8^+$ T cells within the proliferating population is significantly increased. (B) Percentage of each subpopulation that is proliferating. The TN and CD8 Subsets have significantly less proliferation at 2years than at 2 months. At 2 weeks post-castration, the TN population has returned to normal young levels of proliferation while the CD8 population shows a significant increase in proliferation. The level is equivalent to the normal young by 4 weeks post-castration. (C) Overall TN proliferation remains constant with age and post-castration, however, the significant decrease in proliferation of the TN1 subpopulation with age, is not returned to normal levels by 4 weeks post-castration (D).

*** = highly significant, $p<0.001$
** = significant, $p<0.01$

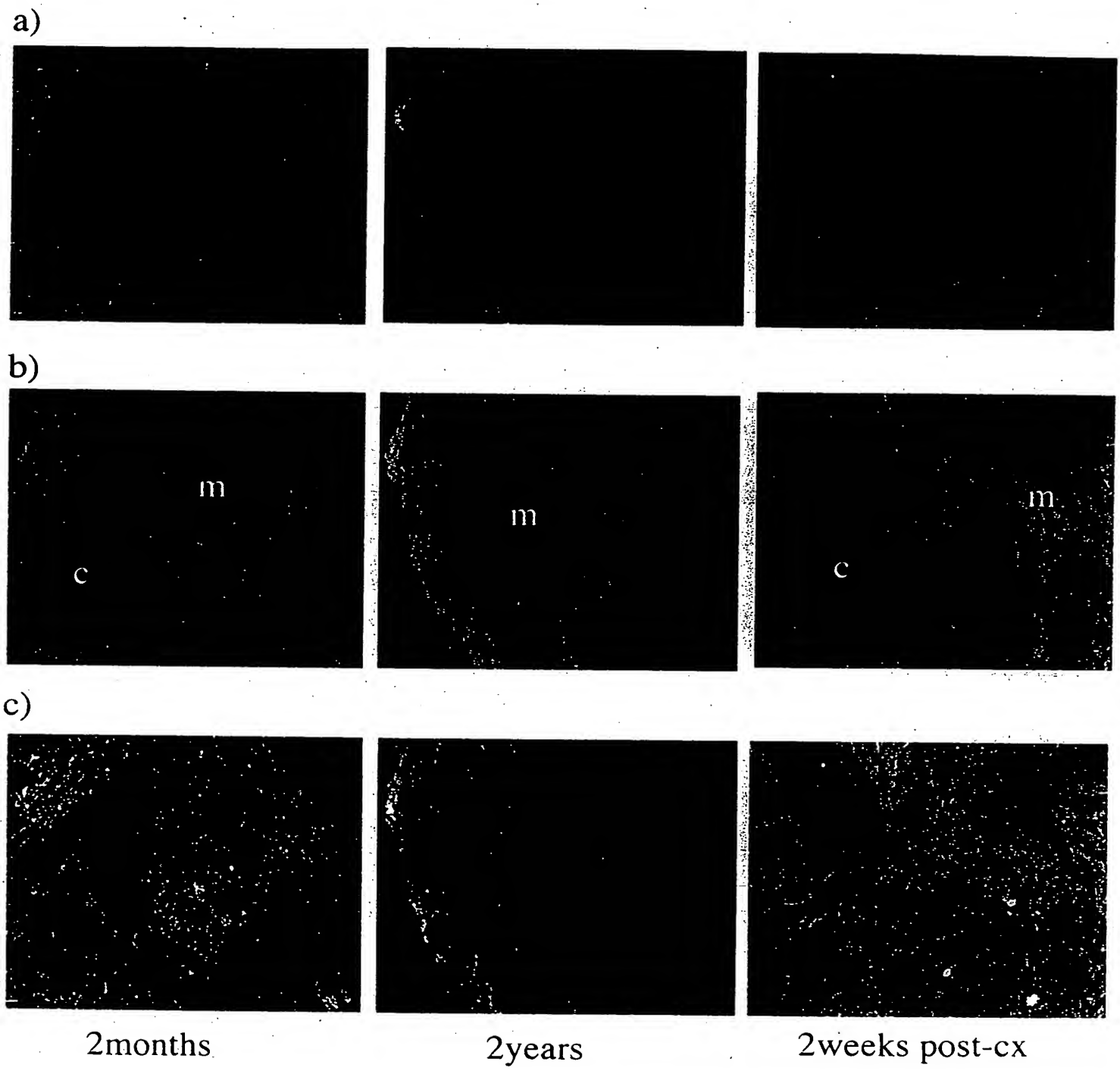

Fig 5.3: Immunofluorescence of thymus from 2 months, 2 years and 2 weeks post-castrated mice. Staining with MTS 44 (a) (cortex); anti-keratin (b) and MTS 44 with anti-keratin (c). Note the substantial decrease in cortical epithelium with age and rejuvenation post-cx.

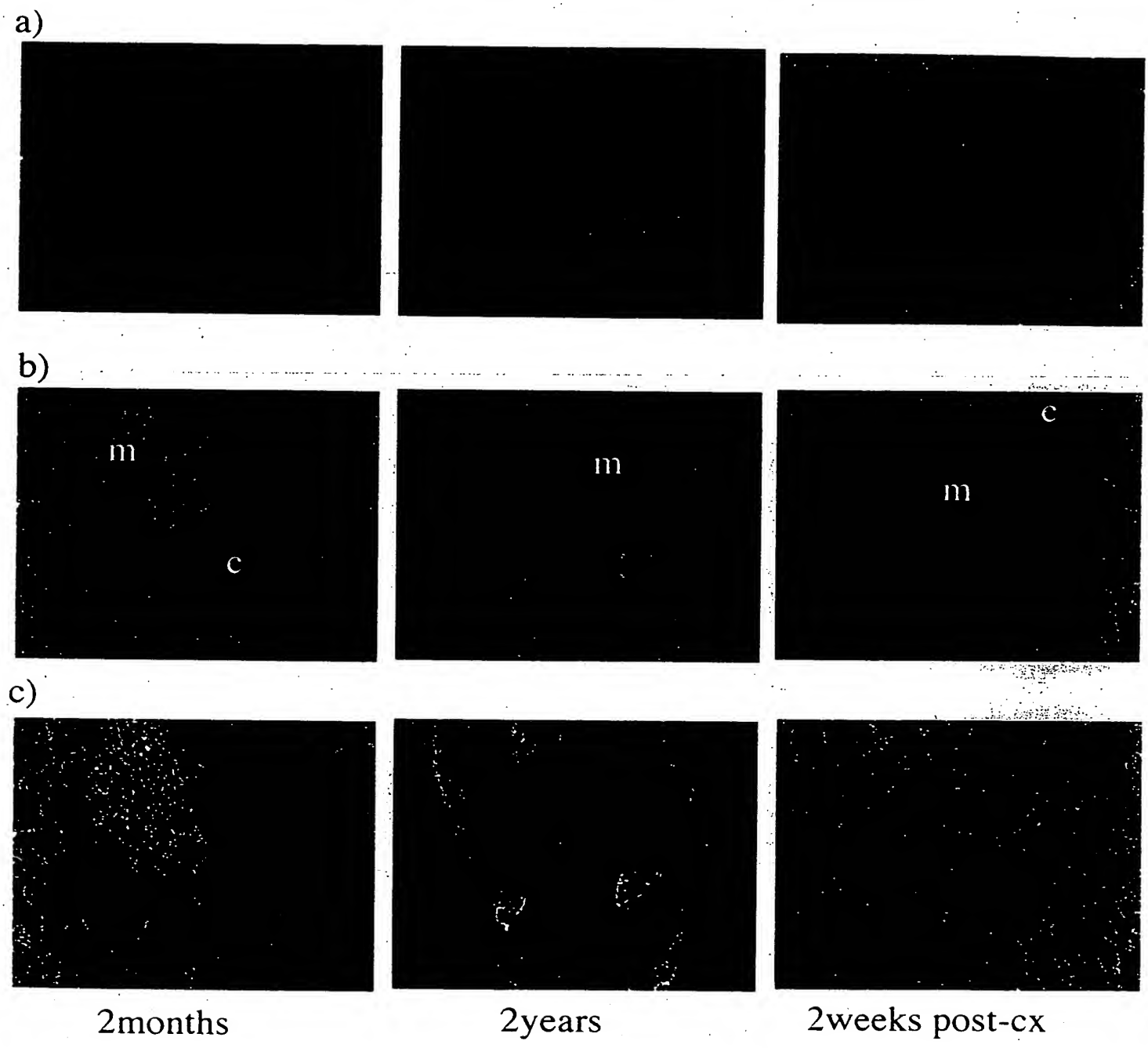

Fig. 5.2: Immunofluorescence of 2 month, 2 years and 2 weeks post-castration mouse thymus Staining with MTS 10 (a) (medulla), anti-keratin (b) and MTS 10 with anti-keratin (c). Note the collapse of the 2 year thymus with indistinct cortico-medullary junction and epithelial disruption (keratin).

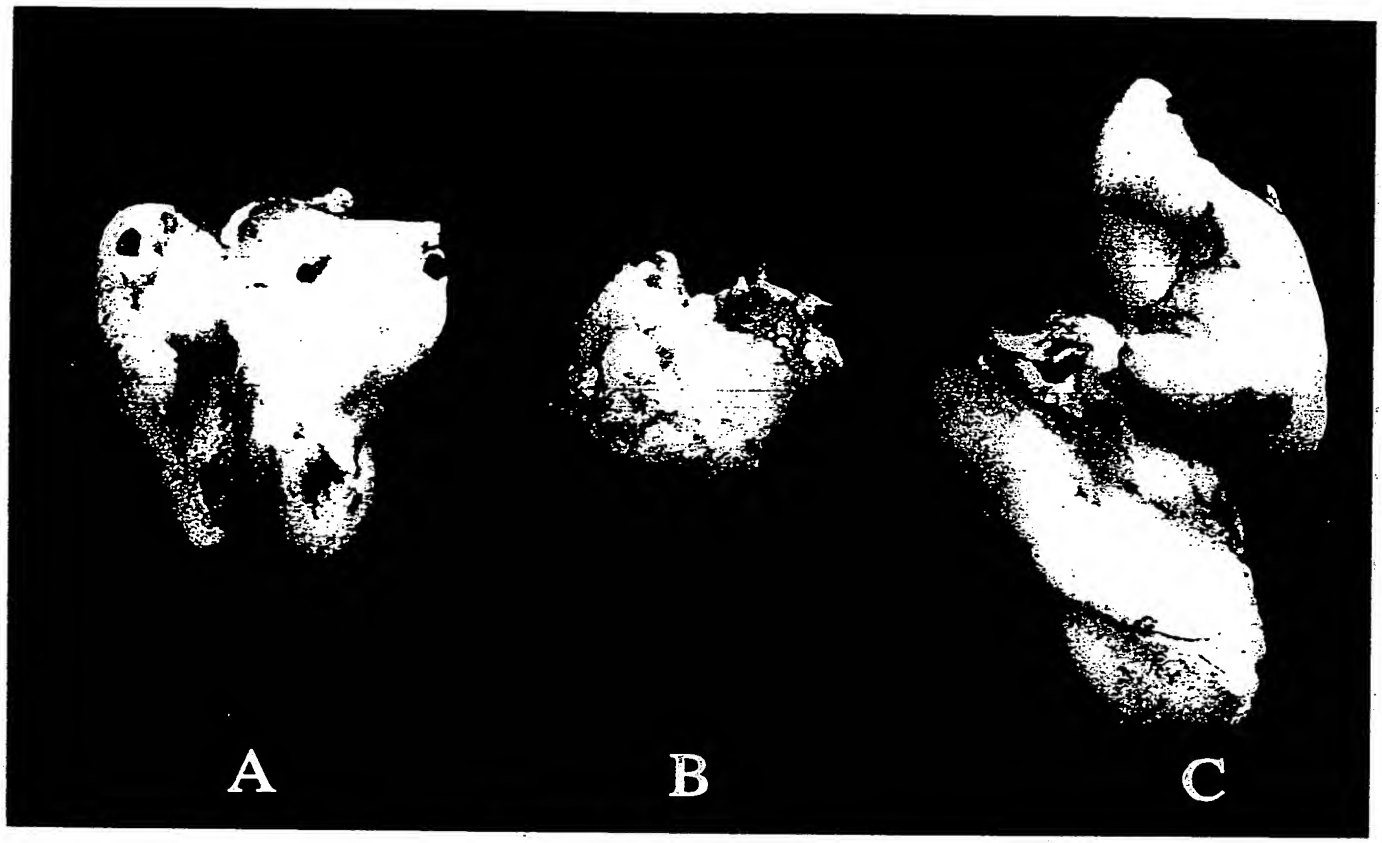

Fig. 5.1. Gross morphology of thymus of (A) 2 year old mouse 4 weeks post-castration: (B) normal 2 year old mouse or (C) normal 2 month old mouse. Note decline in thymic size with age and dramatic increase post-castration.

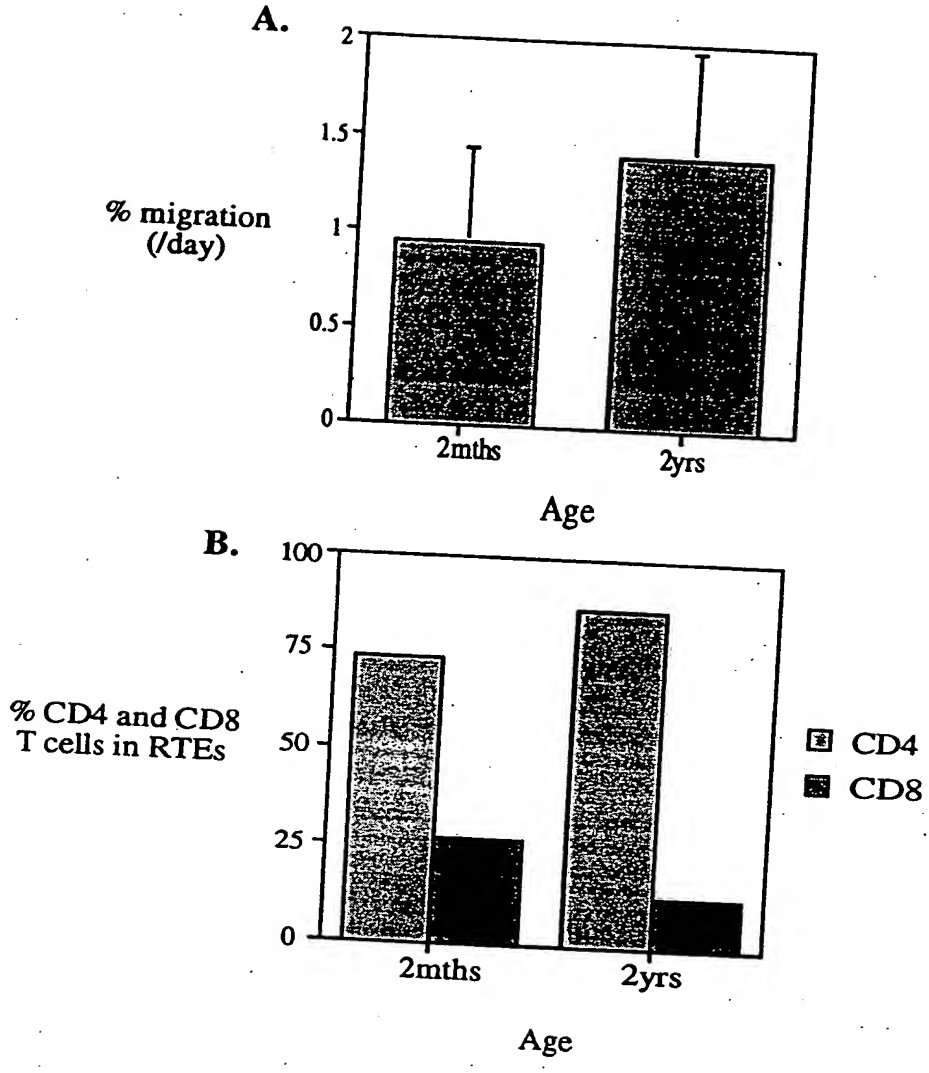

Fig. 6: Migration rates from 1 year and 2 year mice as determined by IT FITC labelling. Young adult migration rates are1% per day. Controls used were non-injected animals. Migration rates remain constant with age.